Isolation of multipotent progenitor cells from human fetal liver capable of differentiating into liver and mesenchymal lineages

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Little is known about the differentiation capabilities of nonhematopoietic cells of the human fetal liver. We report the isolation and characterization of a human fetal liver multipotent progenitor cell (hFLMPC) population capable of differentiating into liver and mesenchymal cell lineages. Human fetal livers (74–108 days of gestation) were dissociated and maintained in culture. We treated the colonies with geneticin and mechanically isolated hFLMPCs, which were kept in an undifferentiated state by culturing on feeder layers. We derived daughter colonies by serial dilution, verifying monoclonality using the Humara assay. hFLMPCs, which have been maintained in culture for up to 100 population doublings, have a high self-renewal capability with a doubling time of 46 h. The immunophenotype is: CD34+, CD90+, c-kit+, EPCAM+, c-met+, SSEA-4+, CK18+, CK19+, albumin−, α-fetoprotein−, CD44h+, and vimentin+. Passage 1 (P1) and P10 cells have identical morphology, immunophenotype, telomere length, and differentiation capacity. Placed in appropriate media, hFLMPCs differentiate into hepatocytes and bile duct cells, as well as into fat, bone, cartilage, and endothelial cells. Our results suggest that hFLMPCs are mesenchymal–epithelial transitional cells, probably derived from mesendoderm. hFLMPCs survive and differentiate into functional hepatocytes *in vivo* when transplanted into animal models of liver disease. hFLMPCs are a valuable tool for the study of human liver development, liver injury, and hepatic repopulation.

epithelial–mesenchymal transition | liver differentiation | liver progenitor cell

The potential applicability of the transplantation of hepatocytes or liver progenitor cells for the treatment of liver diseases has received much attention. Central to the success of this approach is an understanding of hepatic cell lineages and the main steps that give rise to hepatocytes during embryonic development. Liver progenitor cells persist in the adult rodent liver in the canals of Hering (1, 2). These progenitor cells give rise to oval cells, which can generate both hepatocytes and biliary epithelial cells when hepatocytes are unable to mount a proliferative response to injury (3–5).

In rodents, there has been considerable success in isolating precursor cells from the fetal liver and liver epithelial cells or oval cells from adult liver (5–7). Suzuki *et al.* (8, 9) isolated a mouse fetal liver stem cell (c-met+/CD49F+/CD29+/CD45−/CDTER119−) that not only differentiated into hepatocytes and bile duct cells but also was capable of differentiating into intestinal and pancreatic epithelial cells. In humans, isolation of liver stem cell lines has proven more difficult, and to date no unmodified human embryonic liver progenitor cell lines have been reported (10).

Given the diversity of potential hepatoblast precursors described in the mouse, we hypothesized that the human fetal liver might contain intermediate cells in the differentiation pathway from endoderm to hepatoblast. We report here the isolation and characterization of a stable population of human liver progenitor cells from the human fetal liver that does not express liver-specific genes but is able to differentiate into functional hepatocytes and bile duct cells. Of great interest is that these cells, which we named human fetal liver multipotent progenitor cells (hFLMPCs), have features of mesenchymal–epithelial transition; retain multipotent capability to differentiate into fat, cartilage, bone, and endothelial cells; and have repopulation capacity in a mouse model of liver injury.

Results

Derivation of hFLMPCs. In our previous work with primary human fetal hepatocyte cultures, we observed a subpopulation of small blast-like cells that were highly proliferative and expressed markers of liver progenitor cells (11). To isolate and characterize hFLMPCs, we used seven liver specimens obtained from legally aborted first- and second-trimester fetuses between 74 and 108 days of gestation. Using the method described (11), we maintained cells from these livers in primary culture for 3 months to allow enrichment of blast-like cells until they formed a uniform layer over the multilayered cell cultures. Calibrated doses of geneticin were used on passaged cells to selectively arrest fibroblast growth, allowing the proliferating progenitor cell colonies to be mechanically isolated onto feeder layers. We were able to derive hFLMPC colonies from five of seven fetal liver specimens (see Table 1, which is published as supporting information on the PNAS web site). Clones were further derived by serial dilution and maintained for up to 6 months (100 population doublings, 20 passages).

Characterization of hFLMPCs. hFLMPCs grow in a 3D fashion similar to human ES cells (Fig. 1*A*). They are small cells averaging 10 μm in diameter with scanty cytoplasm (Fig. 1*B*). We confirmed that the hFLMPC colonies we derived were clonal by using the Humara X assay. Using an inactivation ratio <0.4 (12–14), all three colonies derived from a heterozygous female specimen (no. 18849) showed nonrandom skewed inactivation of the unmethylated Humara X allele (Fig. 1*C*), confirming clonality.

hFLMPCs Have Self-Renewal Properties Without Evidence of Telomere Shortening. One of the key characteristics of a stem cell population is its ability to self-regenerate. hFLMPCs have an estimated doubling time of 46 h by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) assay (Fig. 1*D*) and demonstrate high BrdU incorporation (see Fig. 7, which is published as supporting information on the PNAS web site). The morphology, immunophenotype, and differentiation potential are identical among hFLMPCs from different passages (data not shown). Cells from P1, P5, and P20 cultures had similar average telomere length that did not shorten, even after >100

Conflict of interest statement: No conflicts declared.

Abbreviations: αFP, α-fetoprotein; HNF, hepatocyte nuclear factor; hFLMPC, human fetal liver multipotent progenitor cell; MSC, mesenchymal stem cell; P*n*, passage *n*; D*n*, day *n*.

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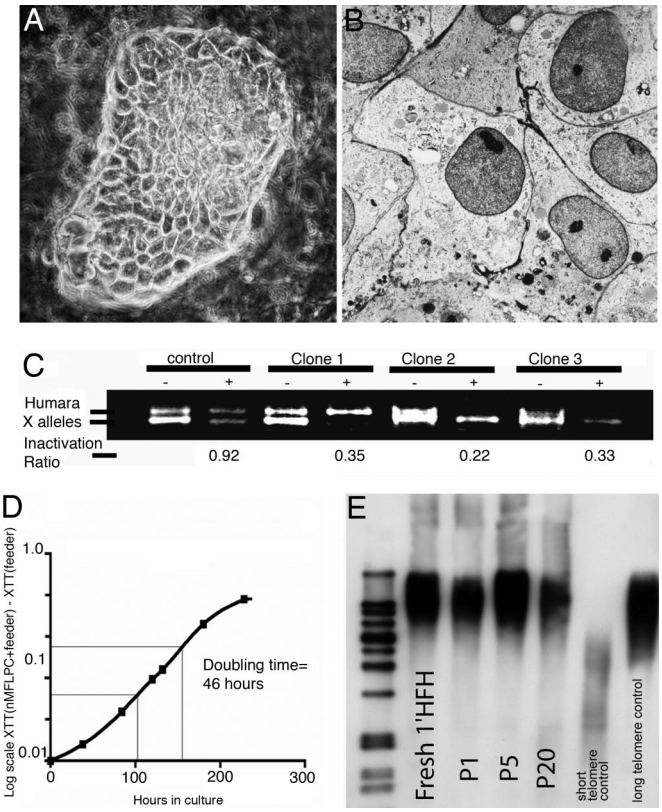

Fig. 1. hFLMPC cultures. (*A*) Phase-contrast micrograph. hFLMPCs grow in heaped-up clusters on NIH 3T3 feeder layers. (*B*) Electron micrograph. hFLMPCs have a high nuclear to cytoplasm ratio with scanty organelles in the cytoplasm and absent bile canaliculi. (*C*) Humara X nested PCR for clonality. Specimen no. 18849 was heterozygous for the Humara X allele (control lanes). DNA extracted from clones 1–3 derived from no. 18849 was subjected to digestion by HhaI (+) or without HhaI (−). The allele inactivation ratios (density ratio of least-intense band to more-intense band, before compared with after digestion) were <0.4 for all three clones, indicating clonality. (*D*) Growth curve of hFLMPCs as determined by XTT activity, revealing a doubling time of 46 h. Proliferation decreases after 10 days as the irradiated feeder layer starts to degenerate. (*E*) Telomere length assay by Southern blotting. Fresh fetal hepatocytes. P1, P5, and P20 hFLMPCs show no apparent shortening of average telomere length, even after 100 population doublings.

population doublings (Fig. 1*E*), suggesting that their self-regenerative potential is durable.

Immunophenotype of hFLMPCs. To determine the immunophenotype of hFLMPCs, we used immunofluorescence or immunohistochemistry, with confirmation by either RT-PCR (when confirming negativity) or immunoblotting (when confirming positivity). hFLMPCs are positive for CD34 (Fig. 2*A*), CD90 (thy-1), c-kit, and SSEA-4 (see Fig. 7). These markers are consistent with a stem-cell signature (15) and have been reported in mouse liver progenitor cells (16). hFLMPCs are also positive for epithelial markers such as EPCAM (Fig. 2*B*), CK18 (Fig. 2*C*), and CK19 (Fig. 2*D*). CK18 and -19 are characteristic markers of hepatocytes and bile duct cells, respectively. Interestingly, hFLMPCs are also positive for the mesenchymal markers CD44h (Fig. 2*E*) and vimentin but are negative for other mesenchymal cell markers, including CD105, CD73, and αSMA (data not shown). Hematopoietic markers such as CD45 and AC133 are also negative (data not shown). hFLMPCs are positive for c-met (Fig. 2*F*) but did not express liver-specific markers such as albumin (Fig. 2*G*), α-fetoprotein (αFP; Fig. 2*H*), or the "liver specification" transcription factors hepatocyte nuclear factor (HNF)1α, HNF3β, and HNF4α (Fig. 4*B*). hFLMPCs are thus a progenitor population within the fetal liver, expressing mixed mesodermal and endodermal markers.

Are hFLMPCs Derived from Other Cells in Fetal Liver Culture? To determine the origin of hFLMPCs, we performed lineage tracing by selective labeling of subpopulation fractions within initial fetal hepatocyte cultures and subsequently deriving hFLMPC colonies (see Fig. 8, which is published as supporting information on the PNAS web site). In cultures in which parenchymal fetal hepatocytes had been GFP-labeled, none of eight derived hFLMPC colonies were GFP-positive. Similarly, in cultures in which mesenchymal stem cell (MSC) fractions were GFP-labeled, none of the six derived hFLMPC colonies were GFP-positive. These results suggest that hFLMPCs are a distinct population in the fetal liver and are unlikely to arise from dedifferentiating parenchymal hepatocytes or transdifferentiating MSCs.

hFLMPCs Are Progenitor Cells for Hepatocytes and Bile Duct Cells. When grown on collagen plates, hFLMPCs flatten into a monolayer, progressively increase in size, and assume a polygonal morphology characteristic of hepatocytes (Fig. 3*A*). Differentiated cells start expressing albumin on day 3 (D3), with 96% of cells becoming strongly positive by D21 (Fig. 3*B*). αFP expression transiently appears at D3–D4 but is no longer detectable by D21 (see Fig. 9, which is published as supporting information on the PNAS web site). These trends were corroborated by immunoblot and RT-PCR (Fig. 4). Correspondingly, albumin in the culture medium was undetectable by ELISA at D0 but increased to 180 ng/ml at D5 and 1.2 μg/ml at D15 (Fig. 3*C*). Differentiated cells store glycogen within the cytoplasm (Fig. 3*D*), and electron microscopic analysis reveals a cytoplasmic organization characteristic of hepatocytes and the formation of bile canaliculi between neighboring cells (Fig. 3*E*). These cells also express α-1-antitrypsin (Fig. 4; also see Fig. 9), and liver-specific cytochrome P450 enzymes inducible by phenobarbital (see Fig. 9). Cytochrome 3A4 production increased 1.6× (immunoblot density quantitation), whereas cytochrome 2B6 activity [pentoxyresorufin-*O*-dependentylase (PROD) assay] was up-regulated 1.4× after phenobarbital treatment. The liver-specific transcription factors HNF1α, HNF3β, and HNF4α, which are not detectable in hFLMPCs, are expressed in differentiated hepatocytes (Fig. 4). Taken together, our data indicate that hFLMPCs can differentiate into functional hepatocytes. These hepatocytes decrease in proliferation upon differentiation but can be maintained in continuous culture up to 6 weeks.

When grown on 3D collagen sandwich gels, hFLMPCs rearrange into cystic and tubular structures by D5 (Fig. 3*F* and see Fig. 9), with cells lining the lumen assuming a columnar shape with tight junctions. Electron microscopy demonstrates long parallel microvilli on the luminal surface of these cells, typical of biliary epithelial cells (Fig. 3*G*). These cells are uniformly positive for markers of biliary cells, including GGT (Fig. 3*H*), CK7 (Fig. 3*I*), and CK19 (Fig. 3*J*).

Expression of Both Mesenchymal and Epithelial Markers in hFLMPCs. hFLMPCs express both epithelial and mesenchymal cell markers. We used immunofluorescence colabeling to determine whether these makers are coexpressed in the same cells, and if so, whether these markers change when hFLMPCs differentiate into hepatocytes. hFLMPCs coexpress both epithelial and mesenchymal markers within the same cell (see Fig. 10, which is published as supporting information on the PNAS web site), and as the expression of liver-specific proteins like albumin increases, mesenchymal markers such as vimentin and CD44h are down-regulated and disappear altogether with complete hepatocyte differentiation. This phenotypic change is characteristic of mes-

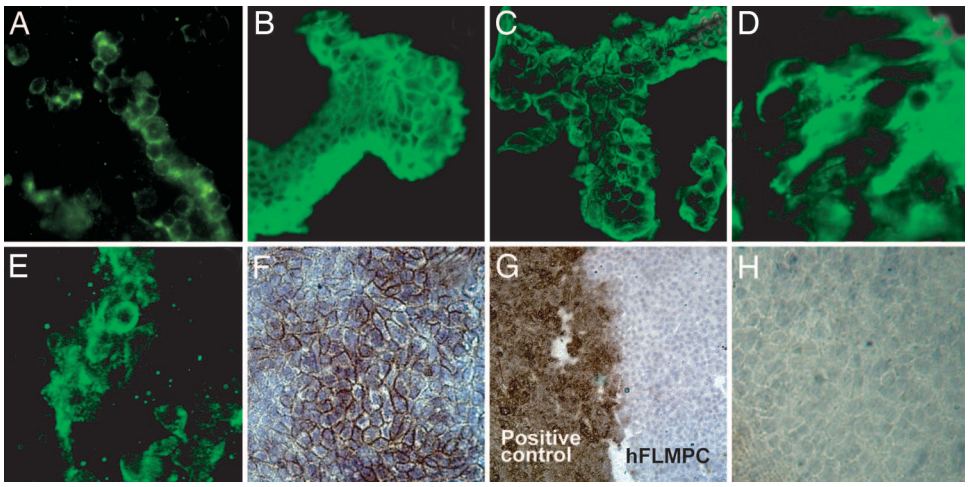

Fig. 2. Characterization of hFLMPCs. (*A–E*) Immunofluorescence and (*F–H*) immunohistochemistry. hFLMPCs are positive for stem cell markers such as CD34 (*A*); epithelial markers such as EPCAM (*B*), CK18 (*C*), and CK19 (*D*); and mesenchymal markers such as CD44h (*E*). They are also positive for c-met (*F*) but do not express liver-specific markers such as albumin (*G*) or αFP (*H*).

enchymal–epithelial transition and suggests that hFLMPCs represent transitional cells between mesenchymal and endodermal lineages.

hFLMPCs Have the Developmental Capacity of MSCs. In view of the expression of mesenchymal markers by hFLMPCs, we investigated whether these cells have the capacity to develop into mesenchymal lineages. In adipose induction media, cells increase in size, and 40% accumulate coalescing vacuoles that stain positive for fat (Oil red O), as opposed to differentiated hepatocytes, which do not survive in fat induction medium. These fat-like cells also express peroxisome proliferator-activated receptor γ2, in contrast to hFLMPCs and differentiated hepatocytes, which do not (Fig. 5 *A* and *B*). In contrast, differentiated hepatocytes do not survive in fat induction medium. Maintained in bone induction medium, cells become elongated with irregular processes, deposit calcium (von Kossa stain), and express osteopontin mRNA (Fig. 5 *C* and *D*). In cartilage induction media, cell clusters form a matrix core containing glycosaminoglycans, which stain positive for safranin O and cartilage-specific collagen type II (Fig. 5 *E* and *F*). In endothelium induction medium containing platelet-derived growth factor (PDGF)-CC (17), hFLMPCs become spindle-shaped and express the endothelial markers CD31 (28%; data not shown) and von Willebrand factor (38%; Fig. 5 *G* and *H*). hFLMPCs can clearly behave like MSCs and, with appropriate stimulation, can give rise to each of the mesenchymal lineage-like cells.

hFLMPCs Can Give Rise to Functional Hepatocytes *in Vivo*. To determine whether hFLMPCs can function as hepatocytes *in vivo*, we transplanted enriched hFLMPCs into immunotolerant Rag2$^{-/-}$ γc$^{-/-}$ mice (18) by using a modified retrorsine/carbon tetra-

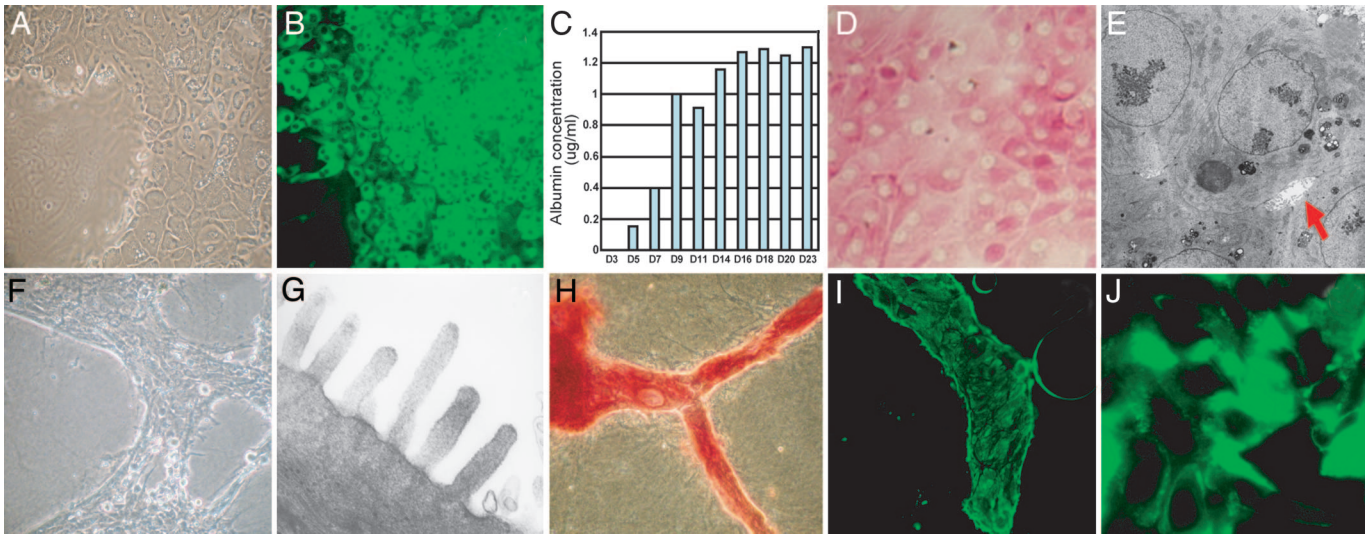

Fig. 3. hFLMPCs are progenitors of hepatocytes and bile duct cells. (*A*) Phase-contrast micrograph. When cultured without feeder layers, hFLMPCs flatten and increase in size to become polygonal cells characteristic of hepatocytes. (*B*) Immunofluorescence. Ninety-five percent of cells at D21 of hepatocyte differentiation are positive for albumin. (*C*) ELISA for human albumin in media increases progressively from D3. (*D*) Differentiated hepatocytes express glycogen deposits (PAS stain). (*E*) Electron micrograph. Differentiated hepatocytes show characteristic formation of bile canaliculi (red arrow). (*F*) Phase-contrast micrographs. When cultured in collagen sandwich gels, hFLMPCs assume a cystic and tubular ductal morphology. (*G*) Electron micrograph. Parallel villi characteristic of biliary epithelial cells are shown. These cells were positive for GGT (*H*, histochemistry), CK7 (*I*, immunofluorescence), and CK19 (*J*, immunofluorescence).

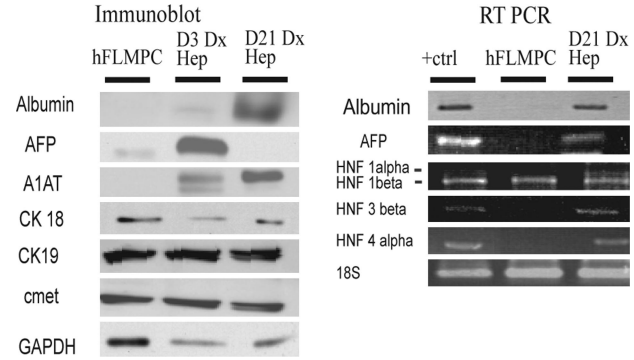

Fig. 4. Expression patterns of hFLMPCs and differentiated hepatocytes. The immunophenotypes of hFLMPCs and differentiated hepatocytes (D3 and D21) were confirmed with immunoblotting (*A*) and RT-PCR (*B*). Albumin, αFP, and α-1-antitrypsin protein are not expressed in hFLMPCs but appear with hepatocyte differentiation. This expression pattern for albumin and αFP is further confirmed with RT-PCR. Similarly, HNF transcripts, including HNF1α, HNF3β, and HNF4α, were absent in hFLMPCs but are present in differentiated hepatocytes. Dx Hep, differentiated hepatocytes; +ctrl, positive control.

chloride model (19). Three of three mice killed 30 days after transplantation had human-specific albumin in the serum (Fig. 6*A*) and expressed human-specific albumin in the liver (Fig. 6*B*). Liver sections of transplanted mice demonstrated clusters of morphologically larger cells (Fig. 6*C*), which stain positive for human-specific albumin (Fig. 6*D*). The degree of repopulation is estimated to be 0.8–1.7% in these mice. These data indicate that hFLMPCs can differentiate into functioning hepatocytes and integrate into the liver parenchyma in an *in vivo* injury model.

Discussion

We report the isolation and characterization of cells from the human fetal liver (hFLMPCs) that are capable of differentiating into hepatocytes and bile duct cells as well as into mesenchymal lineages, including adipose tissue, bone, cartilage, and endothelium. hFLMPCs have a high self-renewal capacity and showed no telomere shortening after 100 population doublings. These cells are discernible as a distinct population at the onset of primary cultures and are highly proliferative (11). Based on our data from mixing experiments with GFP-labeled hepatocytes or MSCs, it is unlikely that hFLMPCs are derived from either of these cell types in culture. Thus hFLMPCs are a unique cell type with stem cell characteristics. To our knowledge, the establishment of clonal cells from human liver that can be maintained in long-term culture in an undifferentiated state and in which directed differentiation to liver and mesenchymal lineages can be controlled at will, has not been previously described.

Although hFLMPCs readily differentiate into hepatocytes and bile duct cells, we did not detect expression of albumin, αFP, or the transcription factors HNF1α, HNF3β, and HNF4α in their undifferentiated state, indicating that these cells are either prehepatoblasts or liver progenitor cells from a parallel differentiation pathway. hFLMPCs coexpress epithelial and mesenchymal markers, and in view of their ability to generate both liver cells and mesenchymal lineages, we consider hFLMPCs to be mesenchymal–epithelial transitional cells and suggest that they have a mesendodermal origin.

It has been hypothesized that mesoderm and endoderm arise from a common bipotential mesendoderm precursor (20). Tremblay and Zaret (21) demonstrated that cells from the ventral midline of the endoderm lip, probably originating from the mesendodermal prechordal plate, give rise to part of the liver bud. Elsewhere in the animal kingdom, 80% of the turtle liver is apparently derived from the mesenchyme (22). Additionally,

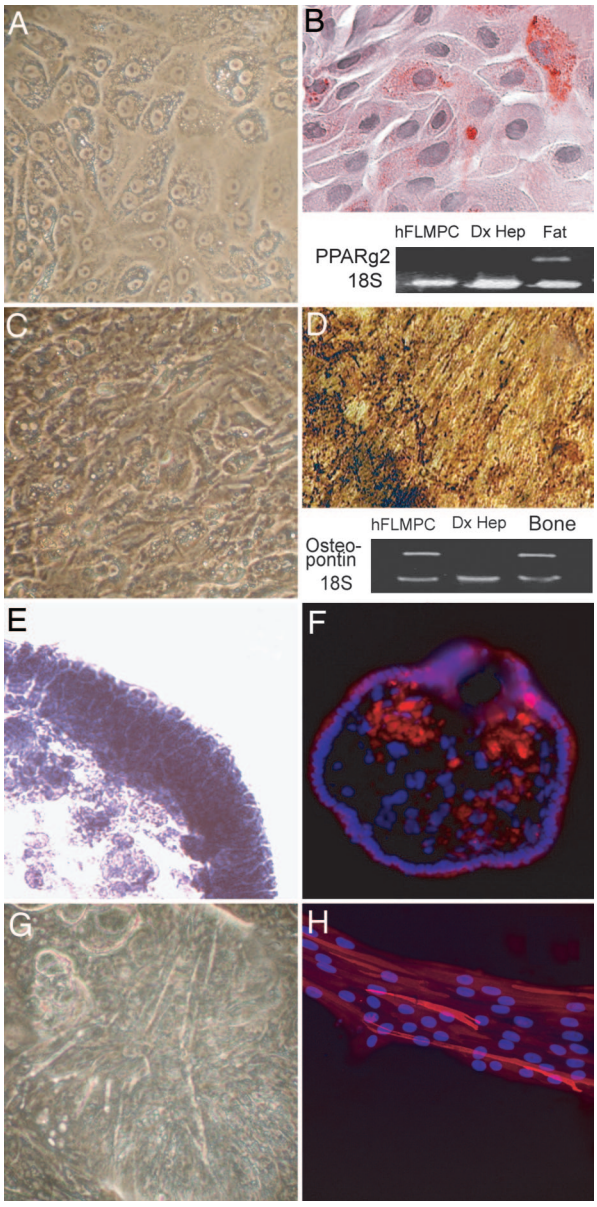

Fig. 5. hFLMPCs have the developmental capacity of MSCs. (*A* and *B*) Adipose tissue induction. hFLMPCs increased in size (*A*) and accumulated vacuoles that stained positive for fat (*B*, Oil red O stain, 40% of cells) and expressed peroxisome proliferator-activated receptor-γ2 (RT-PCR). (*C* and *D*) Bone induction. hFLMPCs assume a longitudinal and interwoven conformation (*C*); deposit calcium into the extracellular matrix, which stains black with von Kossa stain (*D*); and maintain expression of osteopontin (RT-PCR). (*E* and *F*) Cartilage induction. hFLMPC cultures in suspension form a ringed cluster with extracellular matrix in the middle that stains pinkish red with Safranin (*E*) and is positive for collagen II (*F*, red immunofluorescence with blue DAPI nuclear stain). (*G* and *H*) Endothelium induction. (*G*) hFLMPCs become spindle-shaped with formation of linear channels between cells. (*H*) Thirty-eight percent of cells are positive for von Willebrand factor (red immunofluorescence with blue DAPI nuclear stain).

multiple studies have shown that MSCs or mesoderm-related multipotent adult progenitor cells in both mice and humans can differentiate into hepatocytes (23, 24). hFLMPCs appear to have mesendodermal origin, but further fate-map analysis and characterization of liver specification profiles will determine whether they arise from the cells described by Tremblay and Zaret (21) or are from a distinct progenitor niche. Mesenchymal–epithelial

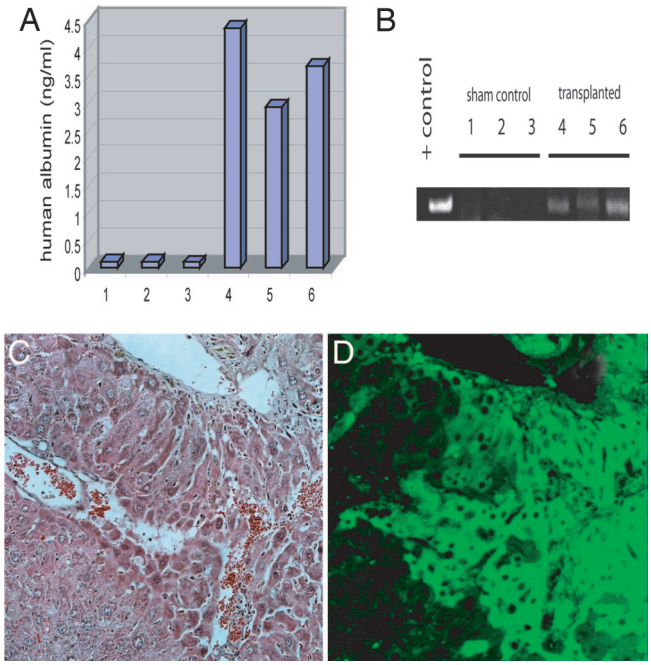

Fig. 6. *In vivo* transplantation of hFLMPCs into Rag2$^{-/-}\gamma c^{-/-}$ mice. (*A*) ELISA for human-specific albumin is positive in serum from mice transplanted with hFLMPCs but not in that of control (sham-operated) mice. (*B*) RT-PCR for human albumin. Livers from transplanted mice express human-specific albumin, in contrast to control mice. This hematoxylin/eosin-stained liver section from a mouse transplanted with hFLMPCs shows a cluster of morphologically different hepatocyte-like cells (*C*), which stain positive for human-specific albumin by immunofluorescence (*D*). The repopulation ratio is estimated to be 0.8%, 1.4%, and 1.7% for the three transplanted mice.

transitional cells are not uncommon in fetal development (25) and have been demonstrated in both rodent neonatal (26) and fetal livers (27), as well as human fetal livers, in which they have been reported to support hematopoiesis (28).

hFLMPCs are clearly different from MSCs or multipotent adult progenitor cells, as described by Jiang *et al.* (29). Their morphology, inherent epithelial markers, and potential to spontaneously become hepatocytes set them apart from MSCs transdifferentiating into epithelial lineages. In our liver differentiation protocols, MSCs from the same fetal livers were used as controls, and they did not become hepatocytes or bile duct cells. Interestingly, hFLMPCs share some morphologic and immunophenotypic characteristics of epithelial cells isolated from patients with subacute hepatic failure (30) and other hepatic diseases (31). They also appear to be similar to bipotential mouse embryonic liver (BMEL) progenitor cells isolated from mouse fetal liver (32). hFLMPCs are derived differently, however, and appear to have a mixed mesendodermal origin rather than the hepatoblast origin of BMEL cells.

In conclusion, we believe we have isolated and characterized human fetal liver progenitor cells with multipotent differentiation potential. hFLMPCs appear to have durable proliferative capability and can be induced to differentiate into functional hepatocytes and biliary duct cells *in vitro*, even after being kept in culture for 6 months. They can differentiate into functional hepatocytes *in vivo*, suggesting liver repopulation potential. We are currently investigating the critical factors that determine their differentiation, proliferation, and lineage transition, as well as the optimal conditions for maintaining differentiated hepatocytes in culture for longer periods of time. We are also exploring the use of this approach to isolate similar cell populations from other embryonic organs, such as pancreas and kidney. We anticipate that hFLMPCs will be a valuable tool to study differentiation pathways in the human liver and may have important therapeutic applications in patients with liver failure.

Materials and Methods

Materials. Basic culture media were purchased from Gibco/Invitrogen; supplements and chemicals were from Sigma Aldrich, unless otherwise specified.

Source and Isolation of hFLMPCs. Human fetal livers were obtained from the Central Laboratory for Human Embryology at the University of Washington, in accordance with a protocol approved by the Institutional Review Board. Primary fetal hepatocyte cultures were raised and maintained in human fetal hepatocyte medium, as described (11).

Cultures were maintained for at least 3 months to allow enrichment of small blast cells before passage with 1 mg/ml collagenase (Boehringer Ingelheim) and 10 units/ml dispase (BD Biosciences, Franklin Lakes, NJ). Geneticin (50 ng/ml; Gibco/Invitrogen) was added to culture media immediately after passage and maintained for 6 days. Colonies of hFLMPCs were then mechanically isolated with microscopic guidance and transferred onto hFLMPC culture plates.

Culture and Cloning. hFLMPCs were cultured on irradiated NIH 3T3 feeder layers (180,000 cells/ml; American Type Culture Collection, Manassas, VA) in plates coated with collagen (Vitrogen, Collagen Corp.)/laminin substrate, maintained with 50% human fetal hepatocyte medium without dexamethasone, 20% FBS, and 30% conditioned medium. Conditioned medium was taken from primary fetal hepatocyte cultures (at least 3 months old) every 48 h and filtered before use. The medium was further supplemented with basic FGF (10 ng/ml) and complex fatty acids (5 ml/liter). Media were changed daily and cultures maintained at 37°C with 6% CO_2. Cloning was performed by serial dilution, and daughter colonies were derived from single- or two-cell clusters on feeder layers, as witnessed under an inverse microscope. Colonies were then passaged every 10 days by using a cut-and-paste technique, as used in human ES cell culture protocols (33).

***In Vitro* Differentiation Protocols.** All differentiation protocols were performed in triplicate with P1–P4 and P8–P12 hFLMPC colonies. Percoll gradient separation was used to isolate human fetal MSCs (34) and parenchymal primary fetal hepatocytes (35) for use as controls. Details of the differentiation protocols are outlined in *Supporting Text*, which is published as supporting information on the PNAS web site.

Characterization of Cells. Immunofluorescence, immunohistochemistry, histochemistry, immunoblotting, RT-PCR, electron microscopy, XTT assay (36), BrdU labeling, and cytochrome P450 assays were based on standard protocols (see *Supporting Text*).

Telomere Length Determination. Telomere length was determined in D4 primary fetal hepatocytes and P1, P10, and P20 hFLMPCs by using the TELOTAGGG telomere length assay (Roche Diagnostics) according to the manufacturer's instructions.

Cell Labeling to Determine Origin of hFLMPCs. To determine the origin of hFLMPCs, parenchymal and MSC fractions from fetal liver were separated by using Percoll density centrifugation (34, 35). The purity of parenchymal and MSC fractions was determined by immunofluorescent expression of albumin or CD44h in random samples of cell fractions in culture at D1 after separation and was ≈90% for both fractions (data not shown). Parenchymal and MSC subpopulations were separately trans-

fected with GFP-labeled lentiviral vectors driven by the EF1α promoter (37, 38) with up to 90% efficiency. Primary hepatocyte cultures were then reconstituted by mixing labeled and nonlabeled fractions, and the presence of GFP expression in derived hFLMPC colonies was determined 3 months later (see *Supporting Text* and Fig. 8 for details).

Determination of Cell Clonality. Clonality of hFLMPCs was determined based on the concept of random X chromosome inactivation by using the Humara assay, as described (39). Clones were derived from a heterozygous female and DNA isolated from P3–P5 colonies. One microgram of DNA was digested with HhaI (Promega) in accordance with the manufacturer's instructions. Nested PCR was then performed as reported (13). Densitometric analysis of ethidium bromide-labeled bands was performed by using SCION IMAGE 4.03 software (Scion, Frederick, MD), and an allele inactivation ratio of <0.4 was indicative of clonality (12–14).

***In Vivo* Transplantation.** Six 4- to 6-week-old Rag2$^{-/-}\gamma^{-/-}$ mice (18) (C57BL/6J $\times$ C57BL/10SgSnAi)-[KO]gc-[KO]Rag2; Taconic Farms) were used for *in vivo* transplantation of hFLMPCs and maintained in accordance with animal care protocols. All mice were treated with weekly retrorsine (60 mg/kg i.p.) for 3 weeks to inhibit replication of native hepatocytes. Three mice then underwent intrasplenic injection with enriched hFLMPCs (3×10^6 cells); the remaining three mice underwent a sham operation (laparotomy). i.p. carbon tetrachloride (0.5 ml/kg diluted 1:10 in olive oil) was injected 1 week later and repeated weekly for 2 more weeks in all mice, and all were harvested 4 weeks after transplantation. Expression of human albumin was determined by serum ELISA, RT-PCR (see Table 2, which is published as supporting information on the PNAS web site), and immunofluorescence.

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